Kongeriget Danmark

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Patent- og Varemærkestyrelsen
Kongeriget Danmark

Patent- og
Varemærkestyrelsen
Erhvervsministeriet

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LIPOLYTIC ENZYME

FIELD OF THE INVENTION

The present invention relates to a nucleic acid sequence encoding a lipolytic enzyme from *Fusarium solani*, as well as a recombinant method of producing the lipolytic enzyme.

BACKGROUND OF THE INVENTION

Lipolytic enzymes (such as lipases and phospholipases) are known to be useful, e.g., in baking and detergents.

US 5990069 discloses a lipase from a strain of *Fusarium solani var. minus*. A lipase/phospholipase from *Fusarium oxysporum* and its sequence are disclosed in WO 98/26057.

SUMMARY OF THE INVENTION

The inventors have isolated a gene encoding a lipolytic enzyme from *Fusarium solani* MUCL 38667 and cloned it into an *E. coli* strain. Accordingly, the invention provides a DNA sequence encoding a lipolytic enzyme.

The nucleic acid sequence of the invention may comprise a nucleic acid sequence which encodes a lipolytic enzyme and comprises:

a) the DNA sequence encoding a mature lipolytic enzyme shown in SEQ ID NO: 1, or

b) an analogue of the DNA sequence defined in a) which

i) has at least 80 % identity with said DNA sequence, or

ii) hybridizes at high stringency with said DNA sequence, its complementary strand or a subsequence thereof.

Other aspects of the invention provide a recombinant expression vector comprising the DNA sequence, and a cell transformed with the DNA sequence or the recombinant expression vector. The invention also provides a recombinant methods of producing the lipolytic enzyme.

A comparison with full-length prior-art sequences shows that the mature amino acid sequence of the lipolytic enzyme from *Fusarium solani* has 66 % identity with the lipase/phospholipase from *Fusarium oxysporum* described above, and the corresponding DNA sequences show 68 % identity.

DETAILED DESCRIPTION OF THE INVENTION

Genomic DNA source

The DNA sequence of the invention may be isolated from *Fusarium solani* MUCL 38667.

MUCL 38667 is available on commercial terms from Mycothèque de l'Université Catholique de Louvain, Place Croix du Sud 3, B-1348 Louvain-la-Neuve, Belgium by referring to US 5990069.

Lipolytic enzyme

The lipolytic enzyme encoded by the DNA sequence of the invention is able to hydrolyze carboxylic ester bonds and is classified as EC 3.1.1 according to Enzyme Nomenclature 1992, Academic Press, Inc. The enzyme has lipase (triacylglycerol lipase) activity (EC 3.1.1.3) and may also have phospholipase activity.

Further properties of the lipolytic enzyme are described in US 5990069.

Recombinant expression vector

The expression vector of the invention typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a selectable marker, a transcription terminator, a repressor gene or various activator genes. The vector may be an autonomously replicating vector, or it may be integrated into the host cell genome.

Production by cultivation of transformant

The lipolytic enzyme of the invention may be produced by transforming a suitable host cell with a DNA sequence encoding the lipolytic enzyme, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

The host organism is preferably a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, e.g. a strain of *Aspergillus*, *Fusarium*, *Trichoderma* or *Saccharomyces*, particularly *A. niger*, *A. oryzae*, *F. graminearum*, *F. sambucinum*, *F. cerealis* or *S. cerevisiae*. The production of the lipolytic enzyme in such host organisms may be done by the general methods described in EP 238,023 (Novo Nordisk), WO 96/00787 (Novo Nordisk) or EP 244,234 (Alko).

Hybridization

The hybridization is used to indicate that a given DNA sequence is analogous to a nucleotide probe corresponding to a DNA sequence of the invention. The hybridization conditions are described in detail below.

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (standard saline citrate) for 10 min, and prehybridization of the filter in a solution of 5 x SSC (Sambrook et al. 1989), 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at approx. 45°C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5 % SDS at a temperature of at least 55°C, more preferably at least 60°C, more preferably at least 65°C, even more preferably at least 70°C, especially at least 75°C.

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using an x-ray film.

Alignment and identity

The lipolytic enzyme and the nucleotide sequence of the invention may have identity to the disclosed sequences of at least 85 %, particularly at least 90 % or at least 95 %, e.g. at least 98 %.

For purposes of the present invention, alignments of sequences and calculation of identity scores were done using a Needleman-Wunsch alignment (i.e. global alignment), useful for both protein and DNA alignments. The default scoring matrices BLOSUM50 and the identity matrix are used for protein and DNA alignments respectively. The penalty for the first residue in a gap is -12 for proteins and -16 for DNA, while the penalty for additional residues in a gap is -2 for proteins and -4 for DNA. Alignment is from the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98).

Lipase activity (LU)

A substrate for lipase is prepared an emulsion of 5 % by volume of tributyrin (glycerin tributyrate) using 0.1 % gum Arabic as emulsifier. The hydrolysis of tributyrin at 30 °C at pH 7 is followed in a pH-stat titration experiment. One unit of lipase activ-

ity (1 LU) equals the amount of enzyme capable of releasing 1 µmol butyric acid/min at the standard conditions. 1 KLU = 1000 LU.

Use of lipolytic enzyme

The lipolytic enzyme of the invention can be used in various industrial application of lipolytic enzymes, e.g. in baking, detergents, diglyceride synthesis (EP 307154), acidolysis, interesterification (WO 8802775), ester hydrolysis, oil degumming (JP-A 2-153997, US 5264367), production of lysolecithin (JP patent 2794574, JP-B 6-087751) and in the process described in PCT/DK 00/00109.

Use in baking

The lipolytic enzyme of the invention can be used in the preparation of dough, bread and cakes, e.g. to improve the elasticity of the bread or cake. Thus, the lipolytic enzyme can be used in a process for making bread, comprising adding the lipolytic enzyme to the ingredients of a dough, kneading the dough and baking the dough to make the bread. This can be done in analogy with WO 9404035 and EP 585988.

Use in detergent

The variant may be used as a detergent additive, e.g. at a concentration (expressed as pure enzyme protein) of 0.001-10 (e.g. 0.01-1) mg per gram of detergent or 0.001-100 (e.g. 0.01-10) mg per liter of wash liquor.

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations. In a laundry detergent, the variant may be effective for the removal of fatty stains, for whiteness maintenance and for dingy cleanup. A laundry detergent composition may be formulated as described in WO 97/04079, WO 97/07202, WO 97/41212, PCT/DK WO 98/08939 and WO 97/43375.

The detergent composition of the invention may particularly be formulated for hand or machine dishwashing operations. e.g. as described in GB 2,247,025 (Unilever) or WO 99/01531 (Procter & Gamble). In a dishwashing composition, the variant may be effective for removal of greasy/oily stains, for prevention of the staining /discoloration of the dishware and plastic components of the dishwasher by highly colored components and the avoidance of lime soap deposits on the dishware.

EXAMPLES

Example 1: Cloning of a phospholipase gene from the *Fusarium solani* strain MUCL 38667.

A genomic DNA preparation of the strain MUCL 38667 was made as described in WO 00/24883.

A PCR reaction (96°C 5 min, 30* (94°C 30 sec., 55°C 30 sec, 72°C 1 min), 72°C 5 min) was run using PWO polymerase in 2.5 mM $MgSO_4$ as recommended by the manufacturer (Boehringer Mannheim) with the MUCL 38667 genomic DNA as template, with oligo 161000J1 and 161000J2 (SEQ ID NO: 3 and 4). These oligo'es were designed based conserved sequences in homologous phospholipases.

A fragment of 180 bp was isolated from a 2 % gel. Because the amounts of DNA was very small, a new identical pcr was run, this time using the 180 bp fragment as template rather than MUCL 38667 genomic DNA.

This fragment was cloned into pCR4 using TOPO-cloning as recommended by the manufacture (Invitrogen) and transformed into the *E. coli* strain TOPO10.

DNA preparations where made using the Qiagen minispinprep kit and the clones where sequenced using M13 rev and M13 fwp primer supplied with the TOPO-cloning kit (Invitrogen). Sequence alignment was made to all available DNA sequence using SRS. The 180 bp fragment was identified as originating from a phospholipase gene.

Based on the 180 bp fragment DNA sequence, four primers were designed: 071200J1, 071200j2, 221200J1, 221200J2 (SEQ ID NO: 5-8).

The MUCL38667 genomic DNA (app. 1 µg) was cut with AgeI in a volume of 10 µl and ligated in a volume of 500 µl. The DNA was precipitated in ethanol and redisolved in water. 2 µl of the religated mix was used as template, and oligo 071200J1 and 071200J2 in a PCR reaction using GeneAMP XL PCR kit as recommended by manufacture (Boehringer Mannheim) in a total of 20 µl.

1 µl of this PCR reaction fragments was used as template in a second PCR reaction using nested oligoes 221200J1 and 221200J2 (SEQ ID NO: 5 and 6), which was identical to the above mentioned.

The generated PCR fragment of app. 1500 bp was cloned into pCR4 using TOPO-cloning as recommended by the manufacture (Invitrogen) and transformed into the *E. coli* strain TOPO10.

DNA preparations where made using the Qiagen minispinprep kit and the clones where sequenced using M13 rev and M13 fwp primer supplied with the TOPO-cloning kit (Invitrogen). Sequence alignment was made to all available DNA sequence using SRS. The 1500 bp fragment was identified as originating from the 3' end of a phospholipase gene.

Based on the 1500 bp fragment DNA sequence, one primer was designed: 170101J11 (SEQ ID NO: 9).

The MUCL 38667 genomic DNA (app. 1 µg) was cut with HindIII in a volume of 10 µl and ligated in a volume of 500 µl. The DNA was precipitated in ethanol and redisolved in water. 2 µl of the religated mix was used as template, and oligo 221200J1 and 170101J11 in a PCR reaction using GeneAMP XL PCR kit as recommended by manufacture (Boehringer Mannheim) in a total of 20 µl.

The generated PCR fragment of app. 350 bp was cloned into pCR4 using TOPO-cloning as recommended by the manufacture (Invitrogen) and transformed into the *E. coli* strain TOPO10.

DNA preparations were made using the Qiagen minispinprep kit and the clones where sequenced using T3 and T7 primer supplied with the TOPO-cloning kit (Invitrogen). Sequence alignment was made to all available DNA sequence using SRS. The 350 bp fragment was identified as originating from the 5 'end of a phospholipase gene.

Based on the 350 bp and the 1500 bp DNA sequence, two primers were designed (290101j2 and 020301j1, SEQ ID NO: 10 and 11), thus covering the hole gene.

A PCR reaction (96°C 5 min, 30* (94°C 30 sec., 55°C 30 sec, 72°C 2 min), 72°C 5 min) was run using PWO polymerase in 2.5 mM $MgSO_4$ as recommended by manufacture (Boehringer Mannheim) with the MUCL38667 genomic DNA as template, with oligo 290101J2 and 020301J1 (SEQ ID NO: 10 and 11).

The generated PCR fragment of app. 1100 bp were cloned into pCR4 using TOPO-cloning as recommended by the manufacture (Invitrogen) and transformed into the *E. coli* strain TOPO10.

DNA preparations were made using the Qiagen minispinprep kit and the clones where sequenced using T3 and T7 primer supplied with the TOPO-cloning kit (Invitrogen). Sequence alignment was made to all available DNA sequence using SRS, as well as earlier sequence of the same.

Example 2: Construction of expression vector and transformation into *Aspergillus oryzae*.

The cloned phospholipase gene in the TOPO vector, as well as pJVi9 (WO 97/47746) was cut with the restriction enzymes BamHI and XhoI. The pJVI9 vector and the phospholipase gene were purified from a 1% agarose gel, and ligated o/n.

The ligation was transformed into the *E.coli* strain DH10b, and transformants were isolated.

DNA preparations where made using the Qiagen minispinprep kit and the clones where verified by sequencing using 19670 and 19671 primer (SEQ ID NO: 12 and 13).

The resulting plasmid was transformed into the *Aspergillus oryzae* strain JaI125 (WO 97/35956) using the following method:

Transformation of *Aspergillus oryzae* (general procedure)

100 ml of YPD (Sherman et al., (1981), Methods in Yeast Genetics, Cold Spring Harbor Laboratory) are inoculated with spores of *A. oryzae* and incubated with shaking for about 24 hours. The mycelium is harvested by filtration through miracloth and washed with 200 ml of 0.6 M $MgSO_4$. The mycelium is suspended in 15 ml of 1.2 M $MgSO_4$, 10 mM NaH_2PO_4, pH 5.8. The suspension is cooled on ice and 1 ml of buffer containing 120 mg of Novozym® 234 is added. After 5 min., 1 ml of 12 mg/ml BSA (Sigma type H25) is added and incubation with gentle agitation continued for 1.5-2.5 hours at 37°C until a large number of protoplasts is visible in a sample inspected under the microscope.

The suspension is filtered through miracloth, the filtrate transferred to a sterile tube and overlayed with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH 7.0. Centrifugation is performed for 15 min. at 1000 g and the protoplasts are collected from the top of the $MgSO_4$ cushion. 2 volumes of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$) are added to the protoplast suspension and the mixture is centrifuged for 5 min. at 1000 g. The protoplast pellet is resuspended in 3 ml of STC and repelleted. This is repeated. Finally, the protoplasts are resuspended in 0.2-1 ml of STC.

100 µl of protoplast suspension are mixed with 5-25 µg of p3SR2 (an *A. nidulans* amdS gene carrying plasmid described in Hynes et al., Mol. and Cel. Biol., Vol. 3, No. 8, 1430-1439, Aug. 1983) and 5 µg of the pJVI9-phospholipase plasmid in 10 µl of STC. The mixture is left at room temperature for 25 min. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution are added and carefully mixed. The mixture is left at room temperature for 25 min., spun at 2.500 g for 15 min. and the pellet is resuspended in 2 ml of 1.2M sorbitol. After one more sedimentation the protoplasts are spread on minimal plates (Cove, (1966), Biochem. Biophys. Acta 113, 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked, suspended in sterile water and spread for single colonies.

12 independent transformants from the pJVI9-phorpholipase transformations were isolated on minimal plates (Cove, (1966), Biochem. Biophys. Acta 113, 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM

CsCl to inhibit background growth, and at the same time inoculated into a 96-well microtiter dish containing 200 µl minimal media of 1*vogel, 2% maltose (e.g., Methods in Enzymology, Vol. 17 p. 84) in each well.

After three days of incubation at 34°C, media from the cultures in the microtiter dish were assayed for lipase activity. A 10 µl aliquot of media from each well was added to a microtiter well containing 200 µl of a lipase substrate of 0.018% p-nitrophenylbutyrate, 0.1% Triton X-100, 10 mM $CaCl_2$, 50 mM Tris pH 7.5. Activity was assayed spectrophotometrically at 15-second intervals over a five minute period, using a kinetic microplate reader (Molecular Device Corp., Sunnyvale CA), using a standard enzymology protocol (e.g., *Enzyme Kinetics*, Paul C. Engel, ed., 1981, Chapman and Hall Ltd.) Briefly, product formation is measured during the initial rate of substrate turnover and is defined as the slope of the curve calculated from the absorbance at 405 nm every 15 seconds for 5 minutes.

This procedure was repeated and spores of the best producing transformants after the second re-isolation were stored as a defined transformant.

A. oryzae JaL 125 (WO 97/35956) is derived from *Aspergillus oryzae* IFO 4177 available from Institute for Fermention, Osaka; 17-25 Juso Hammachi 2-Chome Yodogawa-ku, Osaka, Japan, having the alkaline protease gene named "alp" (described by Murakami K et al., (1991), Agric. Biol. Chem. 55, p. 2807-2811) deleted by a one step gene replacement method (described by G. May in "Applied Molecular Genetics of Filamentous Fungi" (1992), p. 1-25. Eds. J. R. Kinghorn and G. Turner, Blackie Academic and Professional), using the *A. oryzae* pyrG gene as marker.

CLAIMS

1. A nucleic acid sequence which comprises:
   a) the partial DNA sequence encoding a mature lipolytic enzyme shown in SEQ ID NO: 1,
   b) an analogue of the sequence defined in a) which encodes a lipolytic enzyme and
      i) has at least 80 % identity with said DNA sequence, or
      ii) hybridizes at high stringency with a complementary strand of said DNA sequence or a subsequence thereof having at least 100 nucleotides,
      iii) is an allelic variant thereof, or
   c) a complementary strand of a), b) or c).

2. A nucleic acid construct comprising the nucleic acid sequence of claim 1 operably linked to one or more control sequences capable of directing the expression of the lipolytic enzyme in a suitable expression host.

3. A recombinant expression vector comprising the nucleic acid construct of claim 5, a promoter, and transcriptional and translational stop signals.

4. A recombinant host cell comprising the nucleic acid construct of claim 3.

5. A method for producing a lipolytic enzyme comprising cultivating the host cell of claim 4 under conditions conducive to production of the lipolytic enzyme, and recovering the lipolytic enzyme.

6. A method for preparing a dough or a baked product made from the dough, comprising adding the lipolytic enzyme of claim 1 to the dough.

7. A dough composition comprising the lipolytic enzyme of claim 1.

8. A detergent composition comprising a surfactant and the lipolytic enzyme of claim 1.

SEQUENCE LISTING

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